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Antioxidant, Antibacterial and Anticoagulant Activities of the Methanolic Extract of *Rhaponticum acaule* Fruit Growing Wild in Eastern Algeria

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ABSTRACT

This work describes the study of the antioxidant, antibacterial and anticoagulant effect of the methanolic extract of *Rhaponticum acaule* fruit (Asteracea family), also known as *Lenzea acaulis* L, is one of the aromatic plants from the beginning of spring flowering from March to May. This spontaneous plant develops in the north of Algeria and commonly called Tafgha. The antioxidant activity of the *Rhaponticum acaule* fruit was determined in vitro by the DPPH test; 1,1-diphenyl-2-picryl-hydrazyl, the obtained results showed that the extract had an effective DPPH scavenging activity with an IC₅₀ value of 0.065 mg/ml. The fruit extract exhibited antibacterial activity against three strains of standard bacteria; *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis* with a high inhibition zone diameter against *Escherichia coli* of 45 mm when the extract was diluted to 50%, *Enterococcus* of 13 mm and *Staphylococcus* of 19 mm for a crude and concentrated extract. The anticoagulant activity of the extract was evaluated in vitro on a displaced plasma by measuring two important parameters; the prothrombin level (PL) and International Normalized Ratio (INR). The results showed that the fruit can be used as a source of anticoagulant compounds to prevent arterial and venous thromboses if 25%, 50% and 75% diluted extracts are used (TP are respectively 38.4%, 33.5% and 24.5%, INR are 1.35, 2.71 and 3.83, respectively). For crude extract concentrated to 100%, it can cause haemorrhage.

Keywords: *Rhaponticum acaule*, Extraction, Antioxidant activity, Antibacterial activity, Anticoagulant activity.

INTRODUCTION

Breast Asteraceae is an important family of flowering plants and considered as the largest of all the families of angiosperms; they contain more than 20 000 species, distributed in 1100 genera, comprising about 13 tribes [1]. It provides food and pharmaceutical plants; One of the typical properties of the asteraceae family is its richness in various natural compounds (terpenoides, flavonoids and alkaloids, a family rich in bitter principles) [2]. *Rhaponticum acaule* belonging to the asteraceae family is a perennial plant, with large leaves of 10-15 cm, arranged in rosette on the ground, green above and white tomentose below. Large capitula of 5-6 cm in diameter are placed in the center of the rosette. All the flowers are tubular with yellowish color [3]. It is a North African plant, common throughout northern Algeria, especially in the sandy areas of the coast, the local names are tafgha or tafraït, these terms are indicated by Ibn El-Baytar which specifies that the plant is acaule and grows in North Africa [4]. *Rhaponticum acaule* has been used for a long time in traditional medicine, after harvest, roots, leaves, fruit and flowers are cleared of debris, washed and then dried in the open air and then finely powdered. Powder roots, combined with egg yolk, are used against pulmonary disease; The roots, sprayed, associated with honey, are aphrodisiac, eupeptic and vulnerary [5]; The powder of the roots is mixed with pure honey, to remedy the pains of the intestine; The fruit is used raw or cooked orally against gastritis [6]. The roots and leaves of Tafgha are among the main species used in the traditional pharmacopoeia against stomach disease, tuberculosis, rheumatism [7]. Gas chromatography-mass spectroscopy of the oil obtained from *Rhaponticum acaule* roots shows that aliphatic alcohols constitute the high class (69.2%), followed by terpenes (5.5%), alkenes 5.2%) and alkynes (4.0%). And even, there are aldehydes, ketones, ethers, alkanes [8].

Analysis of the oil obtained from *Rhaponticum acaule* flowers shows that it is rich in terpenes (43.38%) and in aromatic compounds (14.10%), hydrocarbons and their derivatives (4.85%), Fatty acids and their corresponding esters (7.79%), epoxides and ethers (5.76%) of aldehydes (2.82%) and ketones (1.90%). They also show that the majority constituents are: methyl eugenol (10.14%), eugenol (5.26%), caryophyllene oxide (3.45%), spathulenol (3.12%), Farnesyl acetate (3.07%) and linanol (2.71%) [4]. Phytochemical criblage made it possible to characterize families of chemical components present in the roots of *Rhaponticum acaule*, the results reveal the presence of polyphenols (tannins) and reducing compounds in appreciable quantities. In addition, qualitative tests of fatty acids, oils Saponins, steroids and sterols are positive and indicate the presence of these compounds in small amounts [4]. The phytochemical screening of the aerial part of the *Rhaponticum* (flowers and leaves) informed us about the presence in large quantities of fatty acids, tannins, sterols, steroids and essential oils, saponosides and flavonoids [4].

MATERIALS AND METHODS

Chemicals and reagents

Methanol (99%) was purchased from Biochem Chemopharma Co (Canada), Dimethylsulfoxide (99,9%) from Sigma-Aldrich Co (Switzerland), Acide ascorbique (99,7%), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (99%) were purchased from Merck Co, lyophilized Thromboplastin (Rabbit cerebral tissue) and HEPES buffer from Biolabo.

Instruments

UV-Vis Spectrophotometer (Spectro Scan 80DV), rotary evaporator (Laborota 4002), centrifuge (NF 400), Thrombotimeter 2-channel.

Plant material and extraction

R. acaule was gathered at the flowering stage in March 2016 in the area of Sedrata (North east Algeria), Sedrata is located in the east of Algeria, at an altitude of 811 m, a latitude of 36.1284° and a longitude of 7.53147° (36° 7' 42" North, 7° 31' 53" East). Its climate is warm and temperate. Rain falls mostly in winter, with relatively little in summer. The climate map of Köppen-Geiger classifies the climate as a Mediterranean climate. [9,10].

The plant samples were cut into small pieces and air-dried for several weeks. 60g of the powdered plant tissues were extracted by maceration with 600 ml of methanol while 24 h, the resultant extract was filtered and the filtrate was concentrated under reduced pressure and it was taken to dryness under vacuum and stored at 4°C until tested, Figure (01) presents the sample of *Rhaponticum acaule* fruit before and after drying (Figure 1).



Figure 1: *Rhaponticum acaule* fruit before and after drying.

Evaluation of biological activities**Antioxidant activity**

The antiradical activity of the extract was evaluated by the DPPH assay. This method consists in following the reduction of the free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) by an antioxidant using UV-Visible spectrophotometry, by measuring the decrease in the absorbance at 517 nm caused by presence of an antioxidant. DPPH radical possesses an unpaired electron on a nitrogen bridge atom. In the presence of free radical scavengers, the violet-colored 2,2- diphenyl-1-picrylhydrazyl reduces to a yellow diphenyl-2-picryl hydrazine [11].

The protocol followed is that described by Brand- Williams with slight modifications [12], 1 ml of each methanolic solution of the extract at different concentrations are added to 1 ml of the methanol solution of DPPH (0.1 mM). After homogenization, the mixture is incubated at ambient temperature in the dark. After 30 min of incubation, the absorbance was measured at 517 nm against blank samples lacking scavenger. The reference antioxidant used is ascorbic acid. The capability to scavenge the DPPH radical was calculated using the following equation [13]:

$$\text{DPPH radical scavenging effect (\%)} = [(A_0 - A_s)/A_0] \times 100$$

A_C: Absorbance of control (0.5 ml containing DPPH radical solution without extract).

A_s: Absorbance in the presence of the sample (extract)

Antibacterial activity

The antibacterial activity of the methanolic extract was evaluated by disc diffusion method against 3 selected gram-positive and gram-negative species: *S. aureus*, *E. fecalis*, *E. coli*.

Agar diffusion method

The qualitative antibacterial assay of the *R. acaule* extract was performed by the disk diffusion method [14]. All of the bacterial strains mentioned above were incubated at 37°C. for 24 h by inoculation in Mueller-Hinton broth (M-H). Whatman filter paper No. 1 was used to prepare discs of 5 mm diameter. These disks are placed in a test tube for sterilization in an oven for 45 minutes at 130°C. After dissolution of the nutrient medium (Muller Hinton agar) in an autoclave, it is poured into the Petri dishes; the medium is left until it becomes solid and is dried for another 15 minutes. The three bacterial strains were rubbed, placed in test tubes containing 10 ml of nutrient broth, and then the test tubes were well stirred.

10 drops of each tube are taken and placed in tubes containing physiological water. The slurry is spilled into the Petri dishes, passed 3 times over the same area to make sure they are completely covered after the dishes were dried at 37°C for 15 minutes.

Four test tubes were prepared for each extract, filter paper discs 5 mm in diameter were immersed in the tubes and impregnated with a small amount of extract and then deposited on the surface of the dishes previously seeded by the suspension microbial. After incubation for 24 hours at 37°C. The antimicrobial activity is manifested by the appearance of a halo of inhibition of the microbial growth around the disks containing the extract, so that the results have been deduced in measuring the diameter of the inhibition zone.

Anticoagulant activity

The anticoagulant activity of the *Rhaponticum acaule* L. extract was evaluated *in vitro* on normal displaced plasma and using the global and coagulometric tests, prothrombin level (PL) and International Normalized Ratio (INR).

The Quick time or the prothrombin time is the coagulation time of a recalcified citrated plasma in the presence of tissue factor and phospholipids brought in large excess by the thromboplastin reagent. This time is expressed in seconds, relative to the time obtained for control plasma. Conventionally, it is the percentage expression, called the prothrombin level, which is used. For the monitoring of oral anticoagulant treatments with anti-vitamins K (AVK), expression in INR (International Normalized Ratio) is preferred over the expression in seconds or percent. The expression in INR takes into account the sensitivity of the reagent used and allows to limit the differences observed between two laboratories. INR is a number without unit. [15]. PL and INR are determined by a trombotimeter which directly displays the values of prothrombin level and international Normalized Ratio.

Preparation of platelet poor plasma

Blood from adult healthy volunteer free from medication for at least two weeks and fasted for at least 8h was taken by venipuncture and collected in a plastic tube on an anticoagulant solution of 3.8% sodium citrate (1 volume per 9 volumes of blood) on the day of its use.

The blood is centrifuged for 10 minutes at 3000 rpm to obtain platelet-poor plasma (ppp). The displaced plasma used directly or stored at low temperature (-10°C) until its use.

RESULTS AND DISCUSSION

Antioxidant activity

DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances. In the DPPH assay, the antioxidants are able to reduce the stable radical DPPH to the yellow- colored diphenyl-picrylhydrazine. The method is based on the reduction of DPPH in alcoholic solution in the presence of a hydrogen- donating antioxidant due the formation of the non-radical from DPPH-H in the reaction. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [16].

The antioxidant activity of the extract of *Rhaponticum acaule* L, and the ascorbic acid were determined by the DPPH method. The percent inhibition of DPPH for various concentrations is shown in Figure 2.

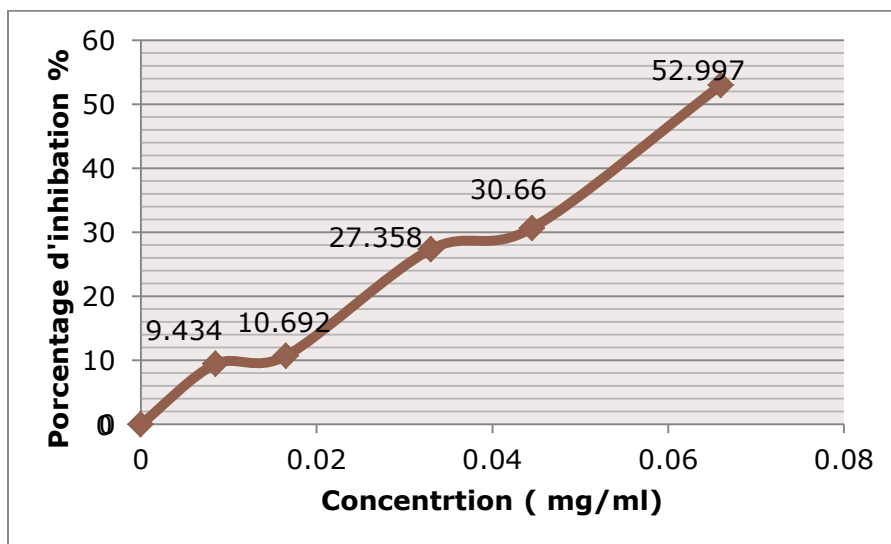


Figure 2: Percentages inhibition of the free radical DPPH at various concentrations of *R. acaule* extract.

Inhibition percentage of the DPPH radical was calculated according to the equation mentioned in the previous section. IC₅₀ value which is the concentration of the scavenger to cause loss of DPPH activity was calculated from the graph plotted between percentage of inhibition and concentration of the sample ($I (\%) = 769.9^{\circ}C + 0.235$, $r^2 = 0.981$).

The degree of discoloration indicates the radical-scavenging potential of the antioxidant [17]. The DPPH radical scavenging activity of the extract increased as concentration increased (Figure 2), methanolic extract exhibited a moderate scavenging activity ($52,997 \pm 1\%$) at a concentration of 0.067 mg /mL.

Antioxidant activity of *R. acaule* fruit can be interpreted by the presence of flavonoids in the aerial part of the plant, these components are one of the most diverse and widespread group of natural compounds and are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties.

Antibacterial activity

The antibacterial activity of the *Rhaponticum acaule* extract is evaluated on three strains (*Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*) after 24 hours of incubation at a temperature of 37°C. The antibacterial power of the extract is obtained by measuring the diameter of the inhibition zone in mm.

Figure 3 illustrates the variations in the diameters of inhibition zones of the bacterial strains which appeared in the presence of *Rhaponticum acaule* extract.

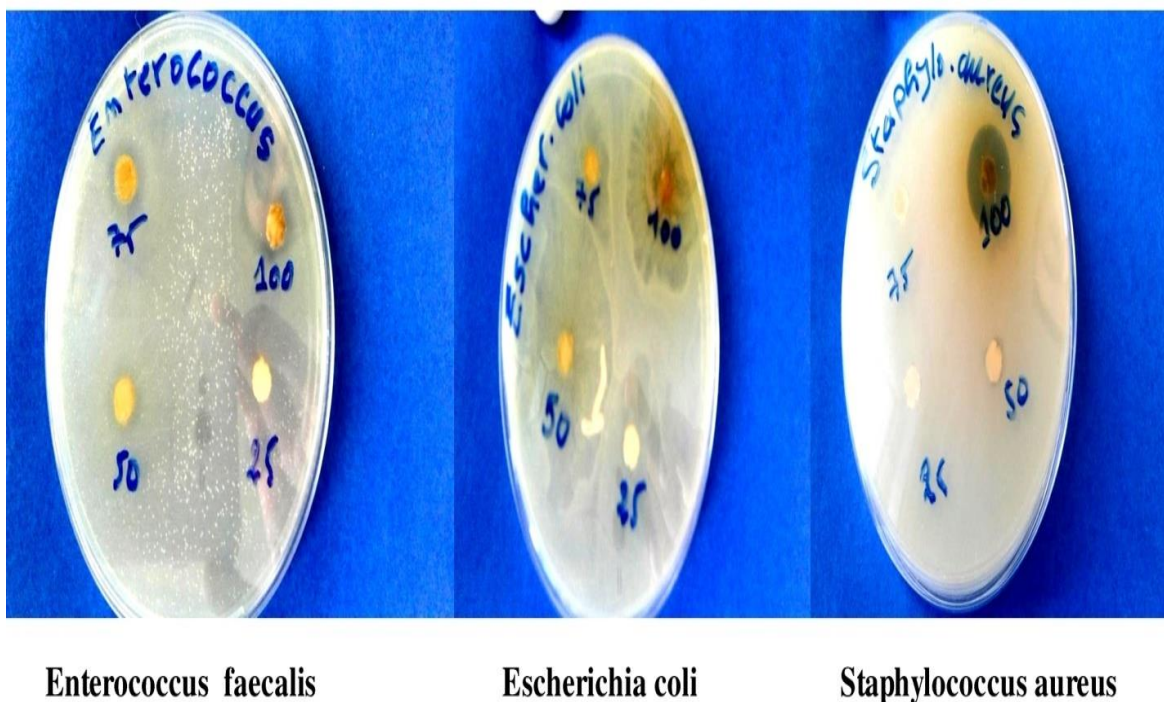


Figure 3: Results of the antibacterial activity of the fruit extract of *Rhaponticum acaule*.

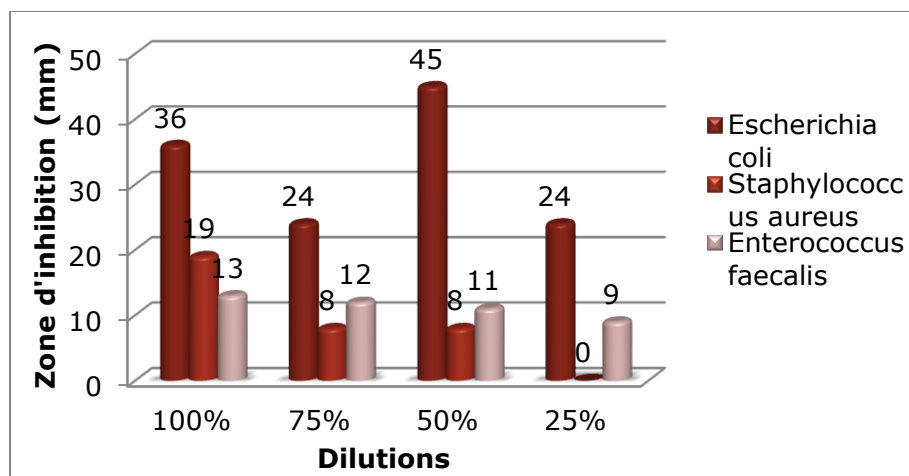


Figure 4: Diameters of the inhibition zones of the bacterial strains growth for different concentrations of the methanolic extract of *Rhaponticum acaule* fruit.

As can be seen in Figures 3 and 4, *Escherichia coli* is very sensitive to the extract for these different concentrations, it exhibits zones of inhibition with diameters of 24 mm, 45 mm and 24 mm, respectively, for dilutions of 75%, 50% and 25%, for *Enterococcus*, it is less sensitive according to their zones of inhibition that appeared in the presence of 75%, 50% and 25% diluted extracts with inhibition diameters of 12 mm, 11 mm and 9 mm, respectively. For *Staphylococcus*, it can be said that the extract has no inhibitory activity on this strain and this is reflected in the small inhibition zones which do not exceed 8 mm.

For the 100% concentrated extract, *Escherichia coli* exhibit an inhibition zone with a large diameter of 36 mm, *Enterococcus*, a diameter of 19 mm and *Staphylococcus* with a diameter of 19 mm.

From this discussion, it can be deduced that *Escherichia coli* is extremely sensitive for the dilution of the extract to 50%, whereas the other strains are sensitive to the 100% concentrated extract.

Anticoagulant activity

Coagulant and anticoagulant system in the body were in a dynamic equilibrium. Discover of the body balance could cause the myocardial infraction, thrombosis, and other blood coagulation with thrombus being a common disease among the old. The activation of the anticoagulation system is an important mechanism for antithrombosis resulting from a series of enzymatic reactions, the blood coagulation process could be divided into three stages: formation of a thrombin activator; activation of thrombin to prothrombin; and the transformation of fibrinogen into fibrin. Integrated by different procedures, the coagulation process could be divided into exogenous and endogenous clotting, so anticoagulant drugs are needed for short-term to treat arterial and venous trombotic disorders and for long-term to prevent of recurrences [18]. Although, heparin and warfarin have been proved as effective anticoagulants, recent studies have revealed some limitations or adverse effects of the drugs, bleeding and alopecia are the major side effects of heparin [19]. So, the search for new substances with anticoagulant and antithrombotic activities is relevant. Medicinal plants have historically been the first source of anticoagulant and antithrombotic molecules like *Careya arborea* (Lecythidaceae family), *Melastoma malabathricum* (Melastomataceae family), *Gloriosa superba* (Lilaceae

family), *Bauhinia forficata* (Leguminosae family), *Eichhornia crassipes* (Pontederiaceae), *Jatropha curcas* L. (Euphorbiaceae), *Synclisia scabrida* (Menispermaceae family), *Porana volubilis* (Convolvulaceae family) and *Erigeron canadensis* (Asteraceae family) [20]. Table 1 shows the prothrombin level and International Normalized Ratio (INR) values at various concentrations of methanolic extract of *Rhaponticum acaule* fruit.

Table 1: Prothrombin level and International Normalized Ratio (INR) values at various concentrations of methanolic extract of *Rhaponticum acaule* fruit.

Concentrations (%)	Prothrombin level (%)	International Normalized Ratio
100	16.6	6.35
75	24.5	3.83
50	33.5	2.71
25	38.4	2.35
Blank	90.80	1.07

Prothrombin is a blood clotting factor protein; it is produced by the liver. In a healthy individual, the TP is between 70 and 100% (for our control blood, we found a TP equal to 90.80%), and the INR is about 1 (for our control blood, found an INR equal to 1.70). A large Quick time or prothrombin time corresponds to a low prothrombin level, the blood being less clotted, it takes longer to coagulate. The INR is then high. Low TP (difficult coagulation) is observed in the following pathological cases: hepatocellular insufficiency (cirrhosis, hepatitis, jaundice) vitamin K deficiency by malabsorption or following AVK (anticoagulant) treatment [16].

Table 2: TP, INR values and corresponding effective treatments

Dilution	TP and effective treatments	INR and effective treatments
25%	38.5%: Treatment of venous thrombosis. Operative prophylaxis	2.35: Treatment of venous thrombosis. Operative prophylaxis
50%	33.5%: Treatment of venous thrombosis. Phlebitis or embolism. Preventive treatment of recurrent thrombosis. Operative prophylaxis	2.71: Treatment of venous thrombosis. Phlebitis or embolism. Preventive treatment of recurrent thrombosis. Operative prophylaxis.
75%	24.5%/Preventive treatment of arterial trombooses. Treatment associated with wearing a cardiac prosthesis.	3.83: Preventive treatment of arterial trombooses. Treatment associated with wearing a cardiac prosthesis.
100%	16.6%: Risk of haemorrhage	6.35: Risk of haemorrhage

By combining data from the literature that relates to TP, INR and the corresponding effective treatments [21] with the experimental TP, INR values found when testing the *R. acaule* extract as an anticoagulant on a healthy blood plasma, can deduce possible and effective treatments for each TP and INR value and for each concentration of the extract, these interpretations are summarized in Table 2.

Thus, the fruit extract of *Rhaponticum acaule* can be used as anticoagulant for dilutions of the extract of 25%, 50% and 75%, on the other hand, for the unreduced (100%) crude extract can cause haemorrhage because that one has a very low TP with a value 16.6% and a high INR with a value of 6.35.

CONCLUSION

Medicinal plants always remain the reliable source of active ingredients known for their therapeutic properties. The development of new therapeutic agents is essential to combat the phenomena of bacterial resistance, oxidative stress and associated diseases, and blood coagulation which cause thrombotic diseases. The present work is devoted to the evaluation of the antioxidant, antibacterial and anticoagulant activity of the methanolic crude extract of the *Rhaponticum acaule* L fruit. Plant harvested in the region of Sedrata wilaya of Souk Ahras (Algeria). Fruit extraction from *Rhaponticum acaule* L was carried out by cold maceration with a high yield (16.88%). Evaluation of the antioxidant activity of the *Rhaponticum acaule* extract by the DPPH free radical scavenging method showed that the extract had an effective antioxidant activity ($IC_{50} = 0.065$ mg / ml). From the values obtained for the diameters of the inhibition zones of the three bacterial strains (*Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*) in the presence of various dilute solutions of the methanolic extract of *Rhaponticum acaule* (100%, 75%, 50% and 25%), it will be noted that the *R. acaule* L extract possesses different antibacterial activities against positive and negative Gram bacteria; The *E. coli* strain, which is extremely sensitive to all concentrations of the extract with inhibition diameters of 24 mm to 45 mm, the *Staphylococcus aureus* strain very sensitive to pure extract with an inhibition diameter of 19 mm and the *Enterococcus faecalis* is sensitive to all dilutions of the extract with zones of inhibition of diameters ranging from 9 mm to 13 mm. The anticoagulant activity of the extract was evaluated *in vitro* on plasma displaced by measuring the time of Prothrombin (TP) and the Ratio Normalized International (INR). The results showed that TP decreases and INR increases. Therefore, this extract could be an alternative against thrombotic diseases.

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